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The Genes for the *Clostridium botulinum* Type G Toxin Complex Are on a Plasmid

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***Clostridium botulinum* type G produces a toxin complex that is composed of neurotoxin, hemagglutinin, and nontoxic nonhemagglutinin. The three genes encoding these proteins were closely linked on a plasmid of about 114 kb (76 MDa) but not on chromosomal DNA. In contrast to the genes of other *C. botulinum* serotypes, the genes encoding type G toxin are on a plasmid.**

Toxins of *Clostridium botulinum* are produced in culture as protein complexes consisting of neurotoxin (BoNT), hemagglutinin (HA), and nontoxic nonhemagglutinin (NTNH) (5, 8, 17, 18, 24). Seven serotypes of botulinum toxin are known, and all of the BoNTs have been purified to homogeneity. The BoNT of *C. botulinum* type G (BoNT/G) was given its type designation because it is antigenically different from the BoNTs of types A through F.

C. botulinum type G strains 89 and 117 were isolated from soils in the Mendoza Province of Argentina (9), and type G was later reported to be present in soils in Switzerland (22) and presumably in necropsy specimens of four adults and an 18-week-old infant (21). Type G toxin is lethal in mice and other vertebrates, including rhesus monkeys (4). Type G toxin has been shown to exist in culture in various complexes with sizes ranging from 300 to 500 kDa (14, 15), but BoNT/G has been extremely difficult to isolate from the complexes without severe losses in activity (14, 15). Recently, BoNT/G was purified to homogeneity, and the light (L) chain was shown to possess the zinc binding motif of zinc endopeptidases (19). BoNT/G is the only toxin serotype that cleaves the two neuronal isoforms of vesicle-associated membrane protein (VAMP)/synaptobrevin at a single Ala-Ala peptide bond (19).

Type G is unusual among *C. botulinum* strains and is physiologically characterized by weak or delayed proteolytic activity, absence of lipase and lecithinase, inability to ferment sugars, infrequent sporulation, and poor production of toxin (4, 20). Type G isolates have the expected 90 to 100% DNA homology, but they also have 85 and 86% homology to two strains of *Clostridium subterminale* and 76% homology to one strain of *Clostridium hastiforme*. It has been suggested that strains producing type G toxin be named *Clostridium argentiense* so that their unique grouping of physiologic characters is emphasized (23). Multilocus enzyme electrophoresis patterns supported the relatedness of *C. botulinum* type G to other nonsaccharolytic clostridia (1).

In a previous study (6, 7), *C. botulinum* type G ceased production of BoNT/G and a bacteriocin when it lost an 81-MDa plasmid during stressful growth at 44°C. This plasmid-toxigenicity relationship provided circumstantial evidence that the BoNT/G gene is on the plasmid. The gene for BoNT/G was recently completely sequenced (3), but this work was not designed to locate the BoNT/G gene. The present communication presents direct evidence that the genes for three proteins of the toxin complex are clustered on a large plasmid.

Culture strains, media, and chemicals. *C. botulinum* type G strains 117 and 89 were from C. Hatheway, Centers for Disease Control and Prevention, Atlanta, Ga. Nontoxigenic *C. subterminale* 28749 was from the American Type Culture Collection (Rockville, Md.). The strains were usually grown in BHIY broth (brain heart infusion [BHI; Difco, Detroit, Mich.] plus 0.5% yeast extract) at 30°C in an anaerobic environment (10% CO_2, 10% H_2, and 80% N_2). The strains were maintained by freezing in cooked meat medium (Difco).

Restriction endonucleases (*Hin*dIII, *Eco*RI, *Xho*I, and *Sma*I) were obtained from Gibco BRL (Gaithersburg, Md.). ATP-dependent DNase, which does not cleave plasmid DNA but acts on linear double-stranded DNA (plasmid safe), and GELase were from Epicentre Technologies (Madison, Wis.). Restriction endonucleases (*Mlu*I, *Rsr*II, and *Nar*I) and reagents for detecting oligonucleotide probes nonradioactively with digoxigenin (Genius System, version 2.0) were from Boehringer Mannheim, Indianapolis, Ind.

DNA purification. Chromosomal DNA was purified as previously described (27). Briefly, cells from young cultures in 200 ml of BHIY broth were suspended in a sucrose-EDTA buffer, pH 8, and stored at −20°C. When needed, the cells in thawed suspensions were lysed by treating them with lysozyme and then with sodium dodecyl sulfate (SDS) plus proteinase K. Sodium perchlorate was added, and the DNA was extracted with phenol-chloroform. The extracted DNA was treated with RNase and extracted twice with phenol-chloroform. The DNA was precipitated with cold ethanol and spooled onto a glass rod, rinsed with ethanol, and vacuum dried at room temperature. The chromosomal DNA was dissolved and further purified by CsCl gradient centrifugation (16). Chromosomal DNA to be used as a template in PCR was additionally purified by electrophoresing in 1% low-melting-point agarose (Bio-Rad, Richmond, Calif.). The region of the gel containing the DNA was transferred into a test tube and dissolved with GELase, using 1 U of enzyme per 600 mg of agarose. The DNA in the digest was incubated for 1 h at 45°C and precipitated with ethanol.

Plasmid DNA was prepared from cultures grown at 30°C in 600 ml of BHIY broth. The medium was inoculated with 30 ml of an overnight culture and incubated to an optical density at 660 nm of 0.6. Cells were harvested by centrifugation, washed

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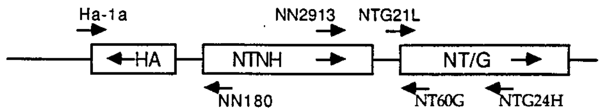

FIG. 1. The location of probes and primers used in this study and the proposed gene orientation for BoNT/G (NT/G), HA, and NTNH.

with 1 volume of 6.7% sucrose–50 mM Tris–1 mM EDTA (pH 8.0), and frozen at −70°C until use. Plasmid DNA was extracted and precipitated by a published method (2, 7). It was then dissolved in 4.5 ml of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) and purified by centrifugation in a CsCl gradient (16). The DNA (1 μg) was treated with plasmid-safe ATP-dependent DNase (5 U) at 37°C for 1 h, which selectively hydrolyzes linear double-stranded DNA (Epicentre Technologies). The DNA preparation was used after holding at 37°C for 1 h.

Probes and primers. Oligonucleotides encoding the amino acid sequences near the N-terminal regions of the light (L) and heavy (H) chains of botulinum type G toxin were synthesized at the Biotechnology Center, University of Wisconsin, Madison. Since the type G toxin gene had not yet been sequenced at the time of this work, the oligonucleotides were based on the amino acid sequences determined by partially sequencing the L and H chains that were purified (11), separated by SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane (12).

These early protein purification results indicated that amino acids 7 through 12 of the L chain could be encoded by TTT AAT TAT AAT GAT CCT. These deduced codons were later found to be identical to those actually present in the BoNT/G gene (3), with the exception that we used GAT instead of the alternate codon GAC. This oligonucleotide (NTG21L) was used as the probe for the BoNT/G gene and as primer 1 in PCR runs. Primer 2 (NTG24H) corresponded to codons for amino acids 8 to 13 of the N terminus of the H chain having the sequence AAA GAA CAA ATC ICC ATT. It differed by three nucleotides from the later-published sequence. The synthesis of oligonucleotide primer NT60G corresponding to nucleotides 60 to 83 of the BoNT/G gene was based on the published DNA sequence (3).

Oligonucleotide Ha-1a, corresponding to a conserved region near Ha-33 (nucleotides 933 to 963) of the type C gene (25), was used as the HA gene probe and as a primer for PCR. Oligonucleotides NN180 (180 to 204) of the NTNH gene (8), corresponding to the N-terminal region of the protein, and NN2913 (nucleotides 2913 to 2937) of the NTNH gene were used as probes in Southern hybridizations and as primers in PCR. The primers used are shown in Fig. 1.

PCR. Regions of the genes for the toxin complex components were amplified by PCR with a Thermal Reactor (Midwest Scientific, St. Louis, Mo.) and the Gene Amp PCR reagent kit (Perkin-Elmer Cetus, Norwalk, Conn.). The 100-μl reaction volume contained 0.3 μg of purified plasmid DNA (maximum available from strain 89) or chromosomal DNA as the template, 50 pmol each of the two primers described earlier, and 2.5 U of *Taq* DNA polymerase. Thirty reaction cycles were carried out with denaturation at 94°C for 1 min, annealing at 42°C for 1.5 min, and extension at 70°C for 2 min (10 min for the final cycle). When NTG21L and NTG24H were used as primers, annealing at 38°C for 2 min was used.

Pulsed-field gel electrophoresis (PFGE). Genomic DNA was prepared by the method of Lin (10) with some modifications. Cultures were grown at 30°C in 10 ml of TPGY (27) broth to an optical density at 660 nm of 0.6. Cells were harvested by centrifugation, washed once with cold TEN buffer (10 mM Tris [pH 7.6], 100 mM EDTA, 150 mM NaCl), and resuspended in 1.5 ml of TEN buffer. The suspension was heated for 10 min at 50°C, mixed with 1.5 ml of 1.5% low-melting-point agarose, and loaded onto a Bio-Rad gel block mold. The cells were lysed by incubating the blocks in EC lysing buffer (6 mM Tris [pH 7.6], 1 M NaCl, 100 mM EDTA, 0.2% deoxycholate, 0.5% Sarkosyl, 20 μg of RNase per ml, 1 mg of lysozyme per ml) overnight at 37°C with gentle shaking. The blocks were incubated in ESP buffer (0.5 M EDTA, 0.5% Sarkosyl, 1 mg of proteinase K per ml [pH 9.0]) for an additional 2 days at 37°C.

For restriction digestion of the DNA, the gel blocks containing the lysed cells were treated twice with 1 mM phenylmethylsulfonyl fluoride in TE buffer (10 mM Tris, 0.1 mM EDTA [pH 7.5]) at room temperature for 2 h. The blocks were then washed three times for 1 h each in TE buffer with gentle shaking and equilibrated on ice for 30 min in 1× restriction enzyme buffer, and each block was placed in 250 μl of 1× restriction enzyme buffer containing 20 U of restriction enzyme and held overnight under conditions recommended by the manufacturer. PFGE was performed at 6 V/cm and 10°C for 22 h by contour-clamped homogeneous electric field electrophoresis (CHEF-DR II; Bio-Rad). Pulses of 5 to 40 s were controlled by a ramp.

Southern hybridizations. The Genius System was used to label the 3′ termini of the probes. NTG21L, corresponding to the N-terminal codons of the L chain, was used as the BoNT/G gene probe. The PCR amplification product was sometimes also used as a BoNT/G gene probe after random primer labeling. NN180 and Ha-1a were used as probes to detect NTNH and HA, respectively.

Southern hybridizations were done by electrophoresing restriction endonuclease-treated DNA samples in 0.7% agarose and transferring the separated DNA fragments to an MSI nylon membrane (Fisher Scientific, Pittsburgh, Pa.) with a Vacuum Blotter (Model 785; Bio-Rad). Hybridization was performed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1.0% blocking reagent–0.1% *N*-lauroylsarcosine–0.02% SDS at 42°C when the oligonucleotide probes were used and at 65°C with the PCR-generated probes.

Isolation and characterization of a plasmid. Plasmid and chromosomal DNAs were purified by procedures using CsCl gradient centrifugation. *C. botulinum* type G strain 117 contained a large plasmid (Fig. 2A) which in both the *Eco*RI and *Hin*dIII digests existed as fragments which totaled about 114 kb. The digested plasmid DNA generated a single 20.5-kb fragment in the *Eco*RI digest and a 1.4-kb fragment in the *Hin*dIII digest that hybridized with the BoNT/G gene probe (Fig. 2B). In contrast to these findings, chromosomal DNAs of type G strain 117 and *C. subterminale* did not hybridize to the probe.

In subsequent experiments, we also used Ha-1a and NN180 as probes in Southern hybridizations with plasmid DNA and chromosomal DNA samples. The probes hybridized with plasmid DNA but not with chromosomal DNA (Fig. 3). A single 3.2-kb fragment in the *Eco*RI digest of the plasmid DNA hybridized with the NN180 and Ha-1a probes.

Only a faint band in the region where plasmid DNA would be expected was found in strain 89. The material in the band did not produce an observable DNA band when electrophoresed on an agarose gel, and Southern hybridizations with the BoNT/G gene probe were negative. Difficulty in recovering plasmid DNA from strain 89 has been previously reported (7).

PCR. The primers shown in Fig. 1 were used in PCR am-

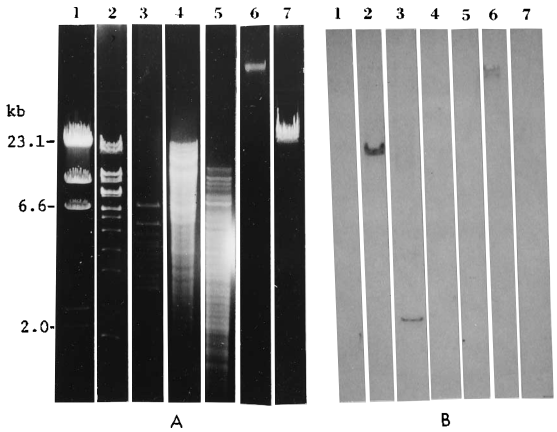

FIG. 2. Southern hybridization of plasmid and chromosomal DNAs purified by CsCl gradient centrifugation. (A) Electrophoretic patterns of plasmid and chromosomal DNAs. (B) Southern hybridization with the BoNT/G gene probe. Lanes: 1, lambda DNA digested with *Hin*dIII; 2, plasmid DNA from strain 117 digested with *Eco*RI; 3, plasmid DNA from strain 117 digested with *Hin*dIII; 4, chromosomal DNA from strain 117 digested with *Eco*RI; 5, chromosomal DNA from strain 117 digested with *Hin*dIII; 6, plasmid DNA from strain 117 (uncut); 7, chromosomal DNA from strain 117 (uncut).

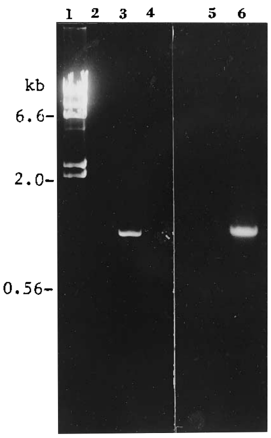

FIG. 4. Agarose gel electrophoresis of PCR amplification products with different templates. The primers used were NTG21L and NTG24H. Lanes: 1, lambda DNA digested with *Hin*dIII; 2, *C. subterminale* 29748 total genomic DNA; 3, plasmid DNA from strain 117; 4, chromosomal DNA from strain 117; 5, chromosomal DNA from strain 89; 6, plasmid DNA from strain 89.

plifications. When NTG21L and NTG24H were used, significant quantities of a product with a size of about 1.3 kb were amplified when the template was plasmid DNA purified from strain 117. Although used in considerably smaller amounts, plasmid DNA of strain 89 also acted as a template so that amplification gave a similar-size product (Fig. 4). These plasmid DNA preparations were also active after being treated with plasmid-safe ATP-dependent DNase. The amplification products hybridized to the BoNT/G gene probe. Since the BoNT/G gene sequence was not known at the time of this work, a probe internal to the two primers was not available. PCR did not yield an amplification product when the template was chromosomal DNA of strains 117 and 89 and total genomic DNA of *C. subterminale*.

Orientation of the genes for the proteins in the toxin complex was examined by PCR. When plasmid DNA from strain 117 or 89 was used as the template and NN2913 and NTG60G were used as the primer pair, a PCR product of 1.3 kb was obtained (Fig. 5). Since NN2913 was a 5′-terminal primer from the sense strand and NTG60G was a 3′-terminal primer from the antisense strand, the results suggested that the NTNH gene is located upstream of the BoNT/G gene and that both genes are transcribed in the same direction. When Ha-1a and NN180 were used as the primer pair, a PCR product of 1.4 kb was obtained. These results indicate that the HA gene is linked to the NTNH gene, but they are transcribed in different directions since both Ha-1a and NN180 are 3′-terminal primers. The probable orientation of the genes is presented in Fig. 1.

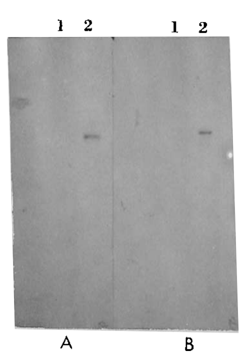

FIG. 3. Southern hybridization analysis for HA and NTNH genes with purified plasmid DNA and chromosomal DNA samples from *C. botulinum* 117. Purified chromosomal and plasmid DNA samples were digested with *Eco*RI and hybridized as described in Materials and Methods. Lanes: 1, chromosomal DNA; 2, plasmid DNA. (A) Hybridization with NN180. (B) Hybridization with Ha-1a.

PFGE. Southern hybridizations done with PFGE used total genomic DNA digested with the rare cutting restriction enzymes *Xho*I, *Sma*I, *Mlu*I, *Rsr*II, and *Nar*I. The corresponding digests of the DNAs of the two toxigenic strains had the same electrophoretic banding patterns, which differed from the banding pattern of *C. subterminale* DNA (Fig. 6A). Southern hybridizations of DNAs of strains 117 and 89 treated with

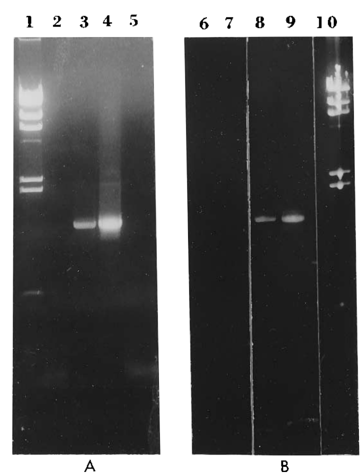

FIG. 5. Agarose gel electrophoresis of PCR amplification products with chromosomal and plasmid templates from *C. botulinum* 117 and 89. The primer pairs used were NN2913 and NT60G (A) and Ha-1a and NN180 (B). Lanes: 1 and 10, lambda DNA digested with *Hin*dIII; 2, chromosomal DNA from strain 117; 3, plasmid DNA from strain 117; 4, plasmid DNA from strain 89; 5, chromosomal DNA from strain 89; 6, chromosomal DNA from strain 117; 7, chromosomal DNA from strain 89; 8, plasmid DNA from strain 117; 9, plasmid DNA from strain 89.

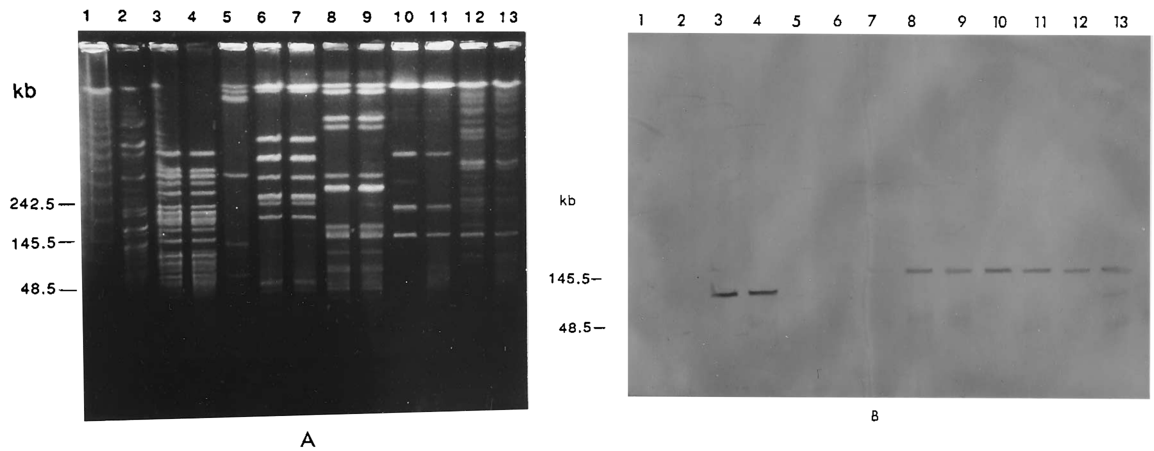

FIG. 6. PFGE and Southern hybridization of genomic DNA. (A) PFGE pattern of genomic DNA. (B) Southern hybridization with L-chain BoNT/G gene probe. Lanes: 1, lambda ladder; 2 and 5, *C. subterminale* 29748; 3, 6, 8, 10, and 12, *C. botulinum* 117; 4, 7, 9, 11, and 13, *C. botulinum* 89. DNA samples were digested with *Xho*I (lanes 2 through 4), *Sma*I (lanes 5 through 7), *Mlu*I (lanes 8 and 9), *Rsr*II (lanes 10 and 11), or *Nar*I (lanes 12 and 13).

*Mlu*I, *Rsr*II, or *Nar*I all showed a DNA piece of about 160 kb (105 MDa) which hybridized to the BoNT/G gene probe (Fig. 6B). The common migration rate suggested that these endonucleases did not cleave the DNA piece carrying the BoNT/G gene.

The DNA fragment in the *Xho*I digests which hybridized to the BoNT/G gene probe was about 108.5 kb, similar to the 114-kb size estimated from the summation of the fragments in the *Eco*RI and *Hin*dIII digests. It is likely that *Xho*I cuts at one site to give a linear product that is unchanged in size but which electrophoreses more rapidly than the closed circular plasmid form.

The earlier report that the gene encoding BoNT/G is located on a plasmid (7) was based on strong, but nevertheless indirect, evidence. The plasmid location is now confirmed by the results of the present study, which show that (i) plasmid DNA purified from *C. botulinum* type G strain 117 hybridizes to a BoNT/G gene probe and (ii) the plasmid DNA but not the chromosomal DNA of the toxigenic strains serves as a template for PCR to amplify an oligonucleotide of the same size (~1.3 kb) as the L chain of the BoNT/G gene. Although it was not previously investigated, we have shown that the genes encoding nontoxic proteins of the type G toxin complex occur as a cluster on the plasmid.

The structural genes of the several botulinum toxin BoNTs as well as those of *Clostridium tetani* have now been located on DNAs from three different sources. The BoNT genes for serotypes A, B, E, and F are chromosomal, those of types C and D and possibly F are located on bacteriophages, and type G and tetanus BoNT genes are located on plasmids (7, 13). The type G BoNT gene has the highest homology with the type B BoNT gene (about 58%), although the first is on a plasmid and the latter is on the chromosome. The clustering of the genes for the BoNT and nontoxic proteins may contribute to their natural transfer to nontoxigenic clostridia (27).

A gene encoding an NTNH has recently been shown to be closely associated with the BoNT genes of types A, B, C, E, and F (5, 8, 26). We have shown that the NTNH gene is on the plasmid carrying the BoNT/G gene. The orientation and same direction of transcription suggest that the NTNH gene and BoNT/G structural gene are closely linked and may constitute an operon. Nucleotide sequencing of the promoter and regulatory regions are needed to affirm this hypothesis.

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